OsLRR-RLK1, an early responsive leucine-rich repeat receptor-like kinase, initiates rice defense responses against a chewing herbivore

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Summary

- Plants are constantly exposed to a variety of environmental stresses, including herbivory. How plants perceive herbivores on a molecular level is poorly understood. Leucine-rich repeat receptor-like kinases (LRR-RLKs), the largest subfamily of RLKs, are essential for plants to detect external stress signals and may therefore also be involved in herbivore perception.
- Here, we employed RNA interference silencing, phytohormone profiling and complementation as well as herbivore resistance assays to investigate the requirement of an LRR-RLK for initiating rice (*Oryza sativa*) -induced defense against the chewing herbivore striped stem borer (SSB) *Chilo suppressalis*.
- We discovered a plasma membrane-localized LRR-RLK, OsLRR-RLK1, whose transcription is strongly up-regulated by SSB attack and treatment with oral secretions of *Spodoptera frugiperda*. *OsLRR-RLK1* acts upstream of mitogen-activated protein kinase (MPK) cascades, and positively regulates defense-related MPKs, and WRKY transcription factors. Moreover, *OsLRR-RLK1* is a positive regulator of SSB-, but not wound-elicited levels of jasmonic acid and ethylene, trypsin protease inhibitor activity and plant resistance towards SSB.
- OsLRR-RLK1 therefore plays an important role in herbivory-induced defenses of rice. Given the well documented role of LRR-RLKs in the perception of stress-related molecules, we speculate that OsLRR-RLK1 may be involved in the perception of herbivory-associated molecular patterns.

Key words: *Chilo suppressalis*; defense responses; ethylene; herbivory perception; jasmonic acid; leucine-rich repeat receptor-like kinase; plant-herbivore interactions; rice

Introduction

In response to herbivore attack, plants activate a wide array of defenses which can reduce herbivore damage, including the initiation of phosphorylation-dependent signaling cascades such as mitogen-activated protein kinase (MPK) cascades, induction of defense-related signaling molecule biosynthesis such as jasmonic acid

(JA), salicylic acid (SA) and ethylene (ET), and the accumulation of defensive compounds (Wu & Baldwin, 2010; Erb *et al.*, 2012; Schuman & Baldwin, 2016). In order to activate their defenses, plants can recognize herbivores through damage-associated molecular patterns (DAMPs) as general wounding cues and herbivore-associated molecular patterns (HAMPs) as herbivore-specific cues (Bonaventure, 2012; Acevedo *et al.*, 2015; Schmelz, 2015). Although the specific pattern recognition by plants is well understood for pathogens (Zipfel, 2014; Couto & Zipfel, 2016), and DAMP perception is being unravelled (Choi *et al.*, 2014; Tanaka *et al.*, 2014; Tripathi *et al.*, 2018), the molecular basis of HAMP perception remains largely unknown (Mithofer & Boland, 2008; Gilardoni *et al.*, 2011; Mescher & De Moraes, 2015; Schmelz, 2015; Schuman & Baldwin, 2016).

Leucine-rich repeat receptor-like kinases (LRR-RLKs) have been shown to play a fundamental role in pattern recognition and initiation of downstream responses (Meng & Zhang, 2013; Macho & Zipfel, 2014). LRR-RLKs are characterized by tandem repeats of LRR motifs in their extracellular domains as well as an intracellular serine/threonine kinase domain (Tor *et al.*, 2009). LRR-RLKs have been shown to be involved in plant responses to wounding (Brutus *et al.*, 2010), gamma irradiation (Park *et al.*, 2014), drought (Osakabe *et al.*, 2005), salt (de Lorenzo *et al.*, 2009), heat (Jung *et al.*, 2015) and pathogens (Song *et al.*, 1995; Gomez-Gomez & Boller, 2000). The flagellin-sensitive 2 (FLS2), for instance, can recognize a conserved 22 amino acid epitope (flg22) from bacterial flagellin by its 28 extracellular LRRs (Gomez-Gomez & Boller, 2000; Gomez-Gomez *et al.*, 2001). Similarly, the elongation factor Tu receptor (EFR) can bind to *N*-acetylated 18 amino acid epitope (elf18) of the bacterial elongation factor Tu (ET-Tu) (Kunze *et al.*, 2004). Xa21 in rice (*Oryza sativa*) confers resistance to *Xanthomonas oryzae* pv. *oryzae* via the recognition of the tyrosine-sulfated protein RaxX (Pruitt *et al.*, 2015). LRR-RLKs have also been associated with plant responses to herbivory. Arabidopsis *pepr1*(Pep receptor 1)*pepr2* double mutants for instance show a reduced accumulation of oral secretion (OS)-elicited JA, and a decreased resistance to *Spodopera littoralis* larvae (Klauser *et al.*, 2015). Moreover, AtBAK1 (brassinosteroid insensitive1-associated

receptor kinase 1) is required for green peach aphid (*Myzus persicae*) elicitor-mediated callose deposition and reactive oxygen species (ROS) induction (Prince *et al.*, 2014). Accordingly, *atbak1* mutants are less resistant to the pea aphid (*Acyrthosiphon pisum*) (Prince *et al.*, 2014). In addition, silencing *NaBAK1* in wild tobacco (*Nicotiana attenuata*) attenuates wound- and OS-elicited JA and JA-isoleucine (JA-Ile) levels, but does not affect MPK activity and herbivore performance (Yang *et al.*, 2011). Despite these findings implicating LRR-RLKs in plant responses to herbivory, the underlying molecular mechanisms remain largely unexplored. Furthermore, the role of LRR-RLKs in plant-herbivore interactions in monocotyledons, as well as their potential to increase resistance against chewing herbivores, is unknown.

MPK cascades link cell surface receptors, such as LRR-RLKs, with downstream signaling components (Rodriguez *et al.*, 2010; Meng & Zhang, 2013). Generally, the stimulated receptors initiate the MPK cascades. Once started, the active MPK kinase kinases (MPKKKs or MEKKs) can activate downstream MPK kinases (MPKKs or MEKs), which subsequently activate MPKs through phosphorylation (Pitzschke, 2015). Activated MPKs phosphorylate their substrates, most of which are transcription factors and enzymes, thereby triggering downstream responses (Pitzschke, 2015). In Arabidopsis, the YODA-MKK4/MKK5-MPK3/MPK6 cascade functions at downstream of ERECTA receptor in regulating plant growth and development (Meng *et al.*, 2012). The MEKK1-MKK1/MKK2-MPK4 and MEKK1-MKK4/MKK5-MPK3/MPK6 can regulate immune responses which are activated by FLS2 after perception of flg22 (Asai *et al.*, 2002; Kong *et al.*, 2012). However, whether MEKK1 acts upstream of MKK4/MKK5 remains controversial (Meng & Zhang, 2013). Moreover, in *N. attenuata*, *Manduca sexta* OS can activate NaMEK2 (ortholog of AtMKK4/AtMKK5), wound-induced protein kinase (WIPK) and SA-induced protein kinase (SIPK, orthologs of AtMPK3 and AtMPK6), which have been reported to be involved in herbivore-induced defense responses via JA signaling (Wu *et al.*, 2007; Hettenhausen *et al.*, 2015). Similarly, the rice

OsMEK4-OsMPK3/OsMPK6 cascade positively regulates the JA signaling pathway and resistance to rice herbivores (Wang *et al.*, 2013; Li *et al.*, 2015).

WRKY transcription factors act as activators or repressors in plant defensive signaling webs downstream of MPK cascades (Ishihama & Yoshioka, 2012). WRKYs can be regulated by MPKs at transcriptional and/or post-translational levels (Ishihama & Yoshioka, 2012; Chi *et al.*, 2013; Li *et al.*, 2015). OsWRKY53, for instance, is activated by OsMPK3 and OsMPK6 through transcriptional induction and phosphorylation, thereby conferring rice resistance to both pathogens and herbivores (Chujo *et al.*, 2014; Hu *et al.*, 2015).

Rice, the most widely consumed food crop, suffers heavily from insect pests (Chen *et al.*, 2011). The striped stem borer (SSB) *Chilo suppressalis*, for instance, can bore into and feed on rice stems and causes large annual yield losses (Chen *et al.*, 2011). SSB attack induces a wide variety of defensive signaling pathways including MPKs, WRKYs, JA, SA and ET, which, in turn regulate rice defense responses (Zhou *et al.*, 2009; Zhou *et al.*, 2011; Lu *et al.*, 2014; Hu *et al.*, 2015).

Here, we isolated an SSB-induced LRR-RLK gene, *OsLRR-RLK1*, and characterized the involvement of this gene in herbivore-induced defense responses in rice. *OsLRR-RLK1* encodes a plasma membrane-localized protein and responses differentially to external stimuli. Using a reverse genetics approach, we obtained rice lines (ir-*lrr*) with reduced expression of this gene and showed that it can positively regulate defense-related MPKs, WRKYs as well as the levels of herbivore-induced JA and ET, which subsequently mediated the activity of defensive trypsin protease inhibitors (TrypPIs) and resistance to SSB. Our study reveals that OsLRR-RLK1 is an early responsive component of herbivore-related signaling pathways.

Materials and Methods

Plants and insects

The rice (*Oryza sativa*) genotypes used in this study were cultivar Xiushui 110 wild-type (WT) and transgenic lines of ir-*lrr* (in this study), as-*mpk3* (Wang *et al.*,

2013), as-*mpk6* (Li *et al.*, 2015), as-*aos1* (Hu *et al.*, 2015), as-*lox* (Zhou *et al.*, 2009), as-*acs2* (Lu *et al.*, 2014) and as-*ics1* (Wang, 2012). These genotypes were cultivated hydroponically as described in Hu *et al*. (2015) with the following composition: 1.43 mM NH_4NO_3, 1 mM $CaCl_2$, 0.32 mM $NaH_2PO_4 \cdot 2H_2O$, 0.51 mM K_2SO_4, 1.64 mM $MgSO_4 \cdot 7H_2O$, 7.58 µM $MnCl_2 \cdot 4H_2O$, 15.11 µM H_3BO_3, 0.12 µM $CuSO_4 \cdot 5H_2O$, 0.06 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.12 µM $ZnSO_4 \cdot 7H_2O$, 28.49 µM $FeCl_3 \cdot 6H_2O$ and 56.63 µM citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$). The pH of the nutrient solution was adjusted to 4.5-5.0 (Yoshida *et al.*, 1976). Forty day-old seedlings were individually transferred to 500 ml hydroponic plastic pots, and then used for experiments 3 to 4 d after transplanting. Larvae of the SSB *Chilo suppressalis* larvae were originally obtained from rice fields in Hangzhou, China, and reared as described by Hu *et al*. (2015). All experiments of this study were repeated at least twice.

Isolation and characterization of *OsLRR-RLK1*

The full-length cDNA of *OsLRR-RLK1* was amplified by PCR. The primers LRR-F (5'-TGCAGCAGGCGAGTTTCATGA-3') and LRR-R (5'-CACAAAAAAGAGGGAAACTAA-3') were designed based on the sequence of *OsLRR-RLK1* (accession no. Os06g47650). The PCR products were cloned into the p*EASY*-blunt cloning vector (TransGen) and sequenced.

OsLRR-RLK1 sequence analysis

Structural domain prediction was performed with SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de;(Schultz *et al.*, 1998; Letunic *et al.*, 2015) and Pfam (http://pfam.sanger.ac.uk) databases. Prediction of trasmembrane domains was performed with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) web servers. Prediction of signal peptides was performed using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP). Protein mass was estimated by ExPASy (http://web.expasy.org/compute_pi/, default setting).

Subcellular localization assay

For subcellular localization, the open reading frame of *OsLRR-RLK1* without the termination code was inserted into the pH7YWG2 plasmid to produce the fused OsLRR-RLK1-enhanced yellow fluorescent protein (EYFP) protein (Karimi *et al.*, 2005). The constructed plasmid was transformed into *Agrobacterium tumefaciens* C5851, and co-infiltrated into *Nicotiana benthamiana* leaves with the C5851 containing mCherry plasma membrane marker plasmid (Nelson *et al.*, 2007) at optical density at 600 nm of 0.7: 0.7. Small living pieces of *N. benthamiana* leaves were assayed for fluorescence 72 h after agroinfiltration. EYFP and mCherry fluorescence were observed and photographed by confocal microscopy (Leica TCS SP5). Spot detection and quantification on confocal micrographs were determined by the ImageJ software with Plot Profile function (https://imagej.nih.gov/ij/index.html).

Plant Treatments

For SSB treatment, one pre-starved third-instar SSB larva was placed on the stem of each plant. Typically, SSB larva crawls toward to the bottom portion of stem and chews a hole to feed on the inner tissues of the plant (Fig. S1). The moment the larva started to chew a hole was defined as time point zero for time course experiments. To measure SSB-induced plant responses, 2 cm portions of the stems around the entry hole were harvested at different time points after infestation. Control plants were not infested, and the same stem portions were harvested for analysis (Zhou *et al.*, 2009). For mechanical wounding, the lower portion of plant stems (approximately 2 cm long) was individually pierced 200 times with a sterilized needle. This piercing treatment aimed at mimicking the tissue damage inflicted by SSB. The damaged sections were harvested in a similar manner as for SSB experiments. Control plants were not pierced, and the same stem portions were harvested (Zhou *et al.*, 2009). For OS treatments, we could not rely on SSB OS, as the larvae do not regurgitate. We therefore used *Spodoptera frugiperda* OS. *Spodoptera frugiperda* attacks rice in the field (Pantoja *et al.*, 1986; Stout *et al.*, 2009) and produces OS that contains well-known defense elicitors such as fatty acid conjugates (FACs) (Yoshinaga *et al.*, 2010; Bonaventure *et al.*, 2011). Plants were wounded as described, and 10 µl of *S. frugiperda* OS was

immediately applied to the wound sites (W + *S. frugiperda* OS). OS was collected from third instar *S. frugiperda* larvae that had been feeding on rice leaves for 48 h, and diluted 1:1 in autoclaved Milli-Q water before use. Ten microliters Milli-Q water were applied to the wounds of control plants (W + water). For JA or SA treatments, plants were individually sprayed with 2 ml of JA (100 µg ml^{-1}) or SA solution (70 µg ml^{-1}) in 50 mM sodium phosphate buffer. Controls (Buf) were sprayed with 2 ml of the buffer (Zhou *et al.*, 2009). For MeJA complementation, plant stems were individually treated with 100 µg of MeJA in 20 µl of lanolin paste. For lanolin treatment (+lanolin), plants were treated similarly with 20 µl of pure lanolin (Hu *et al.*, 2015).

QRT-PCR

For QRT-PCR analysis, five independent biological samples were used. Total RNA was isolated using the SV Total RNA Isolation System (Promega, catalog no. Z3100). One microgram of each total RNA sample was reverse transcribed with the PrimeScript RT-PCR Kit (TaKaRa, catalog no. RR014A). The QRT-PCR assay was performed on CFX96 Real-Time system (Bio-Rad). Gene expression levels were calculated using a standard curve method (Wong & Medrano, 2005). Briefly, a linear standard curve was constructed using serial dilutions of a specific cDNA standard, and drawn by plotting the threshold cycle (Ct) against the $\log_{10}$ of the serial dilutions. The relative transcript levels of the target genes in all unknown samples were then determined according to the standard curve. The rice actin gene *OsACTIN* (accession no. Os03g50885) was used as an internal standard to normalize the cDNA concentrations. Primer specificity was confirmed by agarose gel electrophoresis, melting curve analysis, and sequence verification of cloned PCR amplicons. Primer pair efficiency was determined using the above standard curve method and was found to be between 95% and 105%. The primers, amplification efficiency, TaqMan probe sequences used for TaqMan QRT-PCR (*Premix Ex Taq*™ [Probe qPCR]; Takara, catalog no. RR390A), and primer sequences for SYBR Green-based QRT-PCR (SYBR®*Premix Ex Taq*™ II [Tli RNaseH Plus]; Takara, catalog no. RR820A) are

shown in Table S1.

Phylogenetic Analysis

The program MEGA 6.0 was used for the phylogenetic analysis (Tamura *et al.*, 2013). The protein sequences were aligned using the ClustalW method in MEGA 6.0 (pairwise alignment: gap opening penalty 10, gap extension penalty 0.1; multiple alignment: gap opening penalty 10, gap extension penalty 0.2, protein weight matrix using Gonnet). The residue-specific and hydrophilic penalties were on, and the end gap separation and the use negative separation matrix were off. Gap separation distance was 4, and the delay divergence cutoff (percentage) was at 30. This alignment was then used to generate an unrooted tree with statistical tests (parameters for phylogeny reconstruction were neighbor-joining method [Saitou & Nei, 1987] and bootstrap [Felsenstein, 1985], n = 1,000, amino acid, Poisson model, rate among sites: uniform rates gaps/missing, data treatment: complete deletion, traditional tree without modification for graphics) with MEGA 6.0.

Generation and characterization of transgenic plants

A 298-bp cDNA fragment of *OsLRR-RLK1* was inserted into the pCAMBIA-1301 transformation vector to yield an RNA interference (RNAi) construct (Fig. S2). The vector was inserted into Xiushui 110 using *A. tumefaciens*-mediated transformation. The rice transformation, screening of homozygous T_2 plants and identification of the number of insertions followed the same method as described in Zhou *et al*. (2009). Two T_2 homozygous lines (ir-1 and ir-3) were used in subsequent experiments.

MPK activation detection

One-month-old plants of different genotypes were randomly assigned to SSB or wounding treatments (see earlier). Plant stems were harvested at 0, 15, and 30 min after treatments. Total proteins were extracted from pooled stems of five replicates at each time point using the method described by Wu *et al*. (2007). Forty micrograms of total proteins were separated by SDS-PAGE and transferred onto Bio Trace pure

nitrocellulose blotting membrane (PALL). Immunoblotting was performed using the method described previously (Hu *et al.*, 2015). The primary antibodies used were the plant-actin rabbit polyclonal antibody (EarthOx, catalog no. E021080), which is used as a loading control or the rabbit monoclonal anti-phospho-ERK1/2 (anti-pT-E-pY) antibody (Cell Signaling Technologies, catalog no. 4370), which is specific for the activated (phosphorylated) form of the p44/42 MPKs, when catalytically activated by phosphorylation at the Thr-x-Tyr (TXY) motif (Segui-Simarro *et al.*, 2005; Anderson *et al.*, 2011). As a loading control, actin was detected on a replicate blot. Chemiluminescence-based detection (Thermo Scientific, catalog no. 32109) was performed using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Thermo Scientific, catalog no. 31460). The signal intensities of MPKs and loading actin in the immunoblots were quantified by the ImageJ software as described (Wu & Jackson, 2018). The signal intensity of OsMPK3, OsMPK6 or loading actin for the WT sample at 0 min was set to 1. The relative activation or quantity of all other samples at each time point was expressed relative to the WT sample at 0 min.

JA, JA-Ile, SA, and ET analysis

Plants of different genotypes were randomly assigned to SSB or wounding treatments (see above). Plant stems were harvested at 0, 1.5 and 3 h after the start of the treatments. JA, JA-Ile and SA were extracted with ethyl acetate spiked with labeled internal standards ($^{13}C_2$-JA, $^{13}C_6$-JA-Ile and D-SA, each with 100 ng) and analyzed with HPLC-MS/MS system following the method as described in (Lu *et al.*, 2015). For ET analysis, infested and control plants were covered with sealed glass cylinders (diameter, 4 cm; height, 50 cm). ET levels were determined using the method described by (Lu *et al.*, 2006). Each treatment at each time interval was replicated five times.

Analysis of TrypPI activity

The stems of WT plants and transgenic lines were harvested with SSB treatment for 3 d. The TrypPI activity was measured using a radial diffusion assay as described by

(Jongsma *et al.*, 1994; van Dam *et al.*, 2001). Each treatment was replicated five times.

Herbivore resistance experiments

For SSB performance, freshly hatched SSB neonates were introduced to feed on different rice genotypes. Larval mass was measured 12 d after the start of the experiment. Thirty replicate plants from each line and treatment were used. To determine differences in the tolerance of plants to SSB attack, the different genotypes were individually infested with one third-instar SSB larva. The damage levels of plants were checked and photographs were taken.

Data analysis

Differences in transcript levels of genes, concentrations of JA, JA-Ile, SA, and ET, and herbivore performance in different treatments, lines, or treatment times were determined by analysis of variance (ANOVA). When needed, pairwise or multiple comparisons of Least Squares Means (LSMeans) were corrected using the False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995). All analyses were conducted using R 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

Accession Numbers

Sequence data from this article can be found in the Rice Annotation Project under accession numbers *OsLRR-RLK1* (Os06g47650), *OsWRKY70* (Os05g39720), *OsWRKY53* (Os05g27730), *OsWRKY45* (Os05g25770), *OsWRKY24* (Os01g61080), *OsWRKY33* (Os03g33012), *OsWRKY30* (Os08g38990), *OsMEK4* (Os2g54600), *OsMPK3* (Os03g17700), *OsMPK6* (Os06g06090), *OsHI-LOX* (Os08g39840), *OsAOS1* (Os03g55800), *OsICS1* (Os09g19734), *OsACS2* (Os04g48850), and *OsACTIN* (Os03g50885).

Results

OsLRR-RLK1 is an herbivory induced LRR-RLK

Using microarrays, we identified a putative LRR-RLK that was up-regulated after SSB infestation (Zhou *et al.*, 2011), and obtained its full-length cDNA by reverse transcription PCR. The cDNA nucleotide sequence contains an open reading frame (ORF) of 3201 bp encoding a predicted protein of 1066 amino acids with an estimated molecular mass of 116 kDa (Fig. S3). Analysis of the deduced amino acid sequence predicted the presence of an N-terminal extracellular region including a signal peptide and multiple LRR domains, a single transmembrane domain, and a C-terminal cytoplasmic serine/threonine domain (Fig. S3). Based on these characteristics, the gene was named *OsLRR-RLK1* (for *O. sativa* leucine-rich repeat receptor-like kinase 1).

Comparative analysis of the RLK families in Arabidopsis and rice showed that OsLRR-RLK1 belongs to the LRR-Xb subfamily, cluster 45, clade JA (Shiu *et al.*, 2004). Its closest characterized homologs in Arabidopsis were identified as plant peptide containing sulfated tyrosine 1 receptor (PSY1R), phytosulfokine receptor 2, (PSKR2), phytosulfokine receptor 1 (PSKR1), and receptor like protein 2 (RLP2) (Fig.S4 and S5). PSY1R and PSKR1 have been reported to modify the immunity of Arabidopsis to pathogens (Igarashi *et al.*, 2012; Mosher *et al.*, 2013; Shen & Diener, 2013), and we therefore hypothesized that OsLRR-RLK1 may also be involved in rice defenses.

To determine the subcellular localization of OsLRR-RLK1, its coding region was fused to enhanced yellow fluorescent protein (EYFP) at the N-terminal end, and then expressed in *N. benthamiana* leaves under the control of *CaMV 35S* promoter (*35S::OsLRR-RLK1::EYFP*). As the membrane-localized marker AtPIP2A (Nelson *et al.*, 2007), a fluorescent signal was observed at the plasma membrane (Fig. 1). This suggests that OsLRR-RLK1 may contribute to signal transduction as a component of a receptor system in the plasma membrane.

To investigate the regulation of *OsLRR-RLK1*, we examined its expression levels upon different elicitation treatments using quantitative real-time (QRT)-PCR. Compared with basal mRNA levels in non-manipulated stems (Con), *OsLRR-RLK1*

transcript levels were rapidly and strongly increased upon SSB attack (Fig. 2a). Mechanical wounding also increased *OsLRR-RLK1* mRNA levels, but the induction was weaker and slower compared to SSB attack (Fig. 2a, b). Adding *S. frugiperda* OS to the wounds strongly enhanced wound-induced expression of *OsLRR-RLK1* (Fig. 2c). JA treatment only marginally induced the *OsLRR-RLK1* transcript levels, and SA treatment did not (Fig. 2d, and Fig. S6). These data show that *OsLRR-RLK1* is strongly induced by herbivory, and responds strongly to OS and weakly to wounding alone.

OsLRR-RLK1 silencing by RNA interference

To study the function of *OsLRR-RLK1* in herbivore-induced responses in rice, transformed rice plants with reduced expression levels of *OsLRR-RLK1* were generated by *Agrobacterium tumefaciens*-based plant transformation. Two homozygous single insertion *OsLRR-RLK1*-silenced lines (ir-*lrr* lines: ir-1 and ir-3) were selected and used to characterize the role of *OsLRR-RLK1* (Fig. S7). QRT-PCR analysis showed that both the constitutive and SSB-induced transcript levels of *OsLRR-RLK1* in ir-*lrr* lines were reduced 70-80% compared to wild-type (WT) plants (Fig. S8a). The RNAi construct did not co-silence the transcript levels of the genes whose nucleotide sequences have the highest similarity to *OsLRR-RLK1*, e. g. LOC_Os06g47760 (Top identity 92.72%, Top query coverage, 56.66%, rice genome annotation project algorithm), LOC_Os02g05960 (82.42%, 18.23%), LOC_Os02g05980 (82.37%, 18.89%), LOC_Os02g05920 (82.21%, 12.91%), and LOC_Os02g05940 (82.03%, 18.36%) (Fig. S8). The growth and morphology of ir-*lrr* lines were indistinguishable from those of WT plants at all the development stages both in the greenhouse and the field (Fig. S9).

OsLRR-RLK1 regulates SSB-elicited OsMEK4, OsMPK3 and OsMPK6

MPKs are required for rice defense in response to SSB attack (Wang *et al.*, 2013). To determine whether the silencing of *OsLRR-RLK1* changes MPK cascades, we measured the activation and expression levels of *OsMPK3* (also called *OsMPK5*) and

OsMPK6 (*OsMPK1* and *OsSIPK*) in WT and ir-*lrr* plants. *OsMPK3* is the ortholog of *AtMPK3* and *WIPK*, and *OsMPK6* is the ortholog of *AtMPK6* and *SIPK* (Xie *et al.*, 2014). Their activation was determined by immunoblot analysis using an anti-phosphoERK1/2 (anti-pT-E-pY) antibody. This antibody specifically recognizes the phosphorylated residues within MPK activation loop (the so called pT-E-pY motif, where p denotes the phosphorylated residue), which is required for kinase activity. (Segui-Simarro *et al.*, 2005; Anderson *et al.*, 2011; Schwessinger *et al.*, 2015). In WT plants, SSB infestation rapidly and strongly induced the activation of OsMPK3 and OsMPK6. The activation was reduced in ir-*lrr* lines (Fig. 3a, and Fig. S10). Furthermore, SSB infestation rapidly and strongly induced the expression levels of *OsMPK3* and *OsMEK4*, and marginally induced the expression of *OsMPK6* in WT plants (Fig. 3b-d). The expression levels of *OsMPK3* and *OsMEK4* were significantly decreased in ir-*lrr* plants compared with those in WT plants, whereas *OsMPK6* expression was not affected (Fig. 3b-d). To investigate whether OsLRR-RLK1 is regulated by MPKs, *OsLRR-RLK1* expression was measured in *MPK*-silenced plants (as-*mpk3* and as-*mpk6*, Wang *et al*., 2013; Li *et al*., 2015). *OsLRR-RLK1* expression did not differ between WT and *MPK*-silenced plants (Fig. 3e, f). These results show that OsLRR-RLK1 is a positive regulator of MPKs, and probably acts upstream of the MPK signaling pathway.

OsLRR-RLK1 regulates defense-related WRKYs

WRKYs are an important family of transcription factors to regulate plant defenses (Bakshi & Oelmuller, 2014). We have identified that *OsWRKY70*, *OsWRKY53*, *OsWRKY45*, *OsWRKY24* play crucial roles in the modulation of rice defense in response to herbivory (Li, 2012; Hu *et al.*, 2015; Li *et al.*, 2015; Huangfu *et al.*, 2016). Thus, we determined whether *OsLRR-RLK1* regulates the transcript levels of these four WRKYs and two additional defense-related WRKYs: *OsWRKY30* and *OsWRKY33* (Koo *et al.*, 2009; Han *et al.*, 2013). Silencing of *OsLRR-RLK1* greatly attenuated transcript accumulations of *OsWRKY70*, *OsWRKY53*, *OsWRKY45* and *OsWRKY24*, while it significantly enhanced *OsWRKY30* and *OsWRKY33* transcript

levels after infestation with SSB larvae for 15 and 30 min (Fig. 4).

OsLRR-RLK1 regulates SSB-elicited JA, SA and ET biosynthesis

Given that JA, JA-Ile, SA and ET are central mediators of rice defenses against herbivores (Zhou *et al.*, 2009; Zhou *et al.*, 2011; Lu *et al.*, 2014), we tested whether the reduced expression of *OsLRR-RLK1* alters the production of these phytohormones. JA and JA-Ile induction were significantly reduced in ir-*lrr* lines relative to WT plants (Fig. 5a, b). The transcript levels of JA biosynthesis genes *OsHI-LOX* and *OsAOS1* (Zhou *et al.*, 2009; Hu *et al.*, 2015) were also reduced in ir-*lrr* lines (Fig. 5c, d). ET also accumulated in smaller amounts in SSB-infested ir-*lrr* lines (Fig. 5e), which was associated with reduced expression of the ET biosynthetic gene *OsACS2* (Fig. 5f; Lu *et al*., 2014). By contrast, ir-*lrr* lines accumulated significantly higher SSB-induced SA levels (Fig. 5g) and showed higher expression of the SA biosynthesis gene *OsICS1* (Wang, 2012) (Fig. 5h).

Most LRR-RLKs act upstream of hormonal signaling pathways (Antolin-Llovera *et al.*, 2012). To determine if this is also the case for OsLRR-RLK1 in rice, we quantified the expression of *OsLRR-RLK1* in transgenic plants with impaired JA, SA or ET biosynthesis (as-*lox*, Zhou *et al*., 2009; as-*aos1*, Hu *et al*., 2015; as-*ics1*, Wang, 2012; as-*acs2*, Lu *et al*., 2014). The levels of constitutive and induced *OsLRR-RLK1* transcripts in as-*lox*, as-*aos1*, as-*ics1* and as-*acs2* lines were similar to those in WT plants (Fig. 6). Taken together, these results show that OsLRR-RLK1 acts upstream of JA, SA and ET signaling, and regulates the herbivory-induced biosynthesis of these hormones.

OsLRR-RLK1 does not regulate wound-elicited OsMPK3 and OsMPK6 activation and the levels of JA and SA

To further clarify the OsLRR-RLK1 regulation of herbivory-induced defense responses, we analyzed the MPK activation, JA and SA levels, in ir-*lrr* lines and WT plants after mechanical wounding. OsMPK3 was strongly activated at 30 min, while OsMPK6 was slightly induced at 15 min and decreased at 30 min by wounding.

However, in contrast with SSB infestation, the wound-induced MPK activation remained unchanged in ir-*lrr* lines relative to WT plants (Fig. S11a). Similarly, mechanical wounding significantly induced JA, JA-Ile and SA, but the induction of these phytohormones did not differ between ir-*lrr* lines and WT plants (Fig. S11b-d). These results suggest that OsLRR-RLK1 does not regulate wound-induced OsMPK3 and OsMPK6 activation and the JA, JA-Ile and SA production in the absence of an actual herbivore.

Silencing *OsLRR-RLK1* leads to decreased TrypPI activity and rice resistance to SSB

TrypPIs in rice are antidigestive proteins which are strongly induced by SSB and slow down SSB growth (Zhou *et al.*, 2009). To analyze the function of *OsLRR-RLK1* in regulating TrypPIs, we determined the TrypPI activity in ir-*lrr* lines and WT plants 3d after SSB infestation. Compared with WT plants, ir-*lrr* lines showed a decrease of TrypPI activity of 45% (Fig. 7a). Consistently, SSB neonates gained more weight on ir-*lrr* lines than WT plants (Fig. 7b). Furthermore, ir-*lrr* lines were more susceptible to SSB than WT plants: after infestation by a third instar SSB larva for 7d, ir-*lrr* plants had completely died, whereas WT plants only showed mild dead heart symptoms (Fig. 7g).

To determine if the impaired TrypPI activity and rice resistance in ir-*lrr* lines can be rescued by restoring JA-dependent defenses, we treated ir-*lrr* plants with 100 μg methyl jasmonate (MeJA) in lanolin paste. This complementation restored the TrypPI activity to WT levels (Fig. 7c). Meanwhile, SSB larvae feeding on MeJA-treated ir-*lrr* plants exhibited the same performance as the ones feeding on WT plants (Fig. 7d). Moreover, in another experiment, we found that application of pure lanolin did not impair the difference in TrypPI activity and larval performance between ir-*lrr* lines and WT plants (Fig. 7 e and f). These results suggest that the compromised resistance of *OsLRR-RLK1*-silenced plants is a result of reduced JA signaling that leads to a reduction in defense activation, including TrypPI activity.

Discussion

This study identifies OsLRR-RLK1 as an early herbivore-responsive receptor-like kinase that is required for the initiation of rice defenses against a chewing herbivore.

RLKs can be classified on the basis of their extracellular domains (Tor *et al.*, 2009). OsLRR-RLK1 is placed into the LRR-RLK family due to its putative LRRs in the ectodomain. OsLRR-RLK1 shows high sequence similarity to three receptors PSY1R, PSKR2, PSKR1 and one LRR-receptor like protein (RLP) RLP2 in Arabidopsis (Fig. S5). PSY1R and PSKR1 can specifically bind their ligands, the tyrosine-sulfated peptides PSK or PSY1, via LRR domains (Matsubayashi *et al.*, 2002). PSKR2 is the paralog of PSKR1. RLP2 shares high sequence similarity with the receptor CLAVATA2 (CLV2), which can bind the small signaling peptide CLV3 (Wang *et al.*, 2010). Like PSKR1 and RLP2, OsLRR-RLK1 localized at the plasma membrane (Fig. 1). It is therefore plausible that OsLRR-RLK1-LRR binds to early signaling elements that are associated with herbivory, including for instance HAMPs themselves. Identifying the ligands of OsLRR-RLK1 is an exciting prospect of this work.

Plants can specifically distinguish HAMPs and DAMPs to tailor their defense responses (Bonaventure, 2012). In *N. attenuata*, *NaBAK1* transcript levels are quickly and strongly increased after *M. sexta* OS treatment, but only marginally increased by wounding (Yang *et al.*, 2011). In Arabidopsis, the application of OS as well as *S. littoralis* feeding strongly activates the promoters of *PEPR1* and *PEPR2*, whereas wounding alone does not (Klauser *et al.*, 2015). In our study, the transcript levels of *OsLRR-RLK1* were low in non-manipulated WT plants, but rapidly induced at the early stage (at 0.5 h) and strongly induced at the late stage (after 4 h) by SSB attack. The induction by larval OS was much stronger than mechanical wounding alone. Furthermore, *OsLRR-RLK1* regulated SSB-elicited, but not wounding-elicited MPK activation and phytohormone biosynthesis (Fig. 5, and Fig. S11). These results show that *OsLRR-RLK1* specifically responds to herbivory, and regulates herbivory-induced plant defenses.

Our work places the transcriptional induction of *OsLRR-RLK1* upstream of MPK,

503 WRKY and phytohormone signaling. Exogenous JA only marginally induced
504 *OsLRR-RLK1* expression, and SA did not induce the expression of the gene at all (Fig.
505 2). Furthermore, impairing MPK, JA, SA, or ET signaling did not influence
506 *OsLRR-RLK1* induction (Figs. 3 and 6). Thus, the rapid transcriptional induction of
507 *OsLRR-RLK1* occurs independently of MPKs, JA, SA and ET. For instance it is
508 possible that OsLRR-RLK1 activation triggers transcription via a positive feedback
509 loop. In Arabidopsis, *PEPR1* and *PEPR2* are transcriptionally induced by small
510 peptides (*At*Peps), which are produced from damage-/herbivore-responsive *Precursor*
511 *Protein of Plant Elicitor Peptide* (*PROPEP*) genes, which are in turn regulated by
512 *AtWRKY33* (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2010; Logemann *et al.*, 2013).
513 Furthermore, WRKY proteins can directly bind the W-box elements in the promoter
514 of *RLK4* gene to regulate its expression (Du & Chen, 2000). Therefore, the
515 transcriptional induction of *OsLRR-RLK1* by herbivory or wounding may be achieved
516 through yet unidentified WRKY activity.

517 Plant MPK cascades play central roles in amplifying and transducing signals
518 generated by receptors (Meng & Zhang, 2013). In Arabidopsis, for example,
519 *pepr1pepr2* double mutants have markedly reduced expression levels of *MPK3*
520 (Yamaguchi *et al.*, 2010). A loss of *SERK3/BAK1* results in a marked reduction of
521 flg22 and elf18-triggered activation of MPK3 and MPK6 (Heese *et al.*, 2007), and the
522 knock out mutants for *chitin elicitor receptor kinase 1* (*CERK1*) completely lose the
523 ability to activate MPK3 and MPK6 in response to chitin (Miya *et al.*, 2007).
524 Respective CLV receptors possess unique activities for the regulation of MPK6 in
525 Arabidopsis and *N. benthamiana* (Betsuyaku *et al.*, 2011). Here, we found that
526 *OsMPK6* had high constitutive transcript levels and was only slightly induced by SSB
527 infestation, while *OsMPK3* exhibited the opposite effect. Moreover, silencing
528 *OsLRR-RLK1* reduced the expression levels of *OsMEK4* and *OsMPK3*, as well as the
529 activation of OsMPK3 and OsMPK6 (Fig. 3). These data suggest that *OsMPK3* and
530 *OsMPK6* might also be a pair of paralogous genes, like *AtMPK3* and *AtMPK6* in
531 Arabidopsis (Menges *et al.*, 2008), and that OsLRR-RLK1 can activate MPK
532 components upstream of OsMPK3 and OsMPK6. So far, several receptor-MPK

cascades have been reported. For example, cascades composed of CERK1-PBL27-MAPKKK5-MKK4/MKK5-MPK3/MPK6 in Arabidopsis and OsCERK1-OsRLCK185-OsMAPKKK18 (or OsMAPKKKε) -OsMKK4-OsMPK3/OsMPK6 in rice have recently been reported to be involved in chitin signaling (Yamada *et al.*, 2016; Wang *et al.*, 2017; Yamada *et al.*, 2017). Further researches should elucidate which MPK cascades function downstream of OsLRR-RLK1.

MPKs are known to be upstream regulators of WRKY transcription factors, including the ones that are regulated by OsLRR-RLK1 (Fig. 4). It has been reported that OsWRKY70, OsWRKY53, OsWRKY45, OsWRKY33, OsWRKY30 and OsWRKY24 are downstream of MPK cascades (Koo *et al.*, 2009; Li, 2012; Shen *et al.*, 2012; Ueno *et al.*, 2013; Chujo *et al.*, 2014; Hu *et al.*, 2015; Li *et al.*, 2015). OsWRKY70, OsWRKY53, OsWRKY45 and OsWRKY30 can physically interact with and be phosphorylated by OsMPK3 and/or OsMPK6 (Shen *et al.*, 2012; Ueno *et al.*, 2013; Chujo *et al.*, 2014; Hu *et al.*, 2015; Li *et al.*, 2015). In Arabidopsis, after perception by FLS2, flg22 induces WRKY22 and WRKY29 through activation of a MPK cascade composed of MEKK1, MKK4/MKK5, and MPK3/MPK6 (Asai *et al.*, 2002). In rice, upon herbivore or pathogen infestation, OsWRKY53 and OsWRKY70 are phosphorylated and activated by the OsMKK4-OsMPK3/OsMPK6 cascade (Chujo *et al.*, 2014; Li *et al.*, 2015). Therefore, the regulation of defense-related WRKYs probably occurs through MPK cascade which is modulated by OsLRR-RLK1. Additionally, the induction of some WRKYs as well as MPKs and hormone biosynthesis-related genes seems to be delayed after *OsLRR-RLK1*-silencing. This may be caused by functional redundancy with other homologous *RLK* genes or non-complete silencing of *OsLRR-RLK1*.

In *N. attenuata*, NaBAK1 regulates the accumulation of JA in responses to *M. sexta* (Yang *et al.*, 2011). In Arabidopsis, the lack of *PEPR1/PEPR2* receptors leads to reduced production of JA and JA-Ile after the application of *S. littoralis* OS (Klauser *et al.*, 2015). Furthermore, PSKR1 and PSY1R modify plant immunity to pathogens via JA- and SA- mediated signaling pathways (Mosher *et al.*, 2013). Here, we found

563 that OsLRR-RLK1 positively regulated the production of SSB-elicited JA, ET as well
564 as the transcript levels of their biosynthesis-related genes, such as *OsHI-LOX*,
565 *OsAOS1* and *OsACS2*, whereas it negatively influenced the accumulation of SA after
566 SSB infestation, including the transcript levels of SA biosynthesis-related gene
567 *OsICS1* (Fig. 5). Interestingly, it was also observed that expression levels of
568 *OsHI-LOX*, *OsAOS1* and *OsACS2* were initially reduced in ir-*lrr* lines, and then back
569 to WT levels by 90 min after SSB attack, while *OsICS1* showed the opposite effect.
570 This may reflect the antagonistic crosstalk of JA/ET and SA signaling pathways in
571 rice as reported previously (Lee *et al.*, 2004; Qiu *et al.*, 2007; Yuan *et al.*, 2007).
572 Extensive studies have shown that MPKs and WRKYs mediate the biosynthesis of JA,
573 SA, and ET in rice. For example, OsMPK3 positively regulates SSB-elicited JA levels
574 (Wang *et al.*, 2013). OsMPK6 is involved in pathogen-related JA, SA accumulation
575 (Shen *et al.*, 2010). OsWRKY70, OsWRKY53, OsWRKY45, and OsWRKY24 are
576 implicated in herbivore-induced JA, SA and ET biosynthesis (Li, 2012; Hu *et al.*,
577 2015; Li *et al.*, 2015; Hu *et al.*, 2016; Huangfu *et al.*, 2016). OsWRKY33 and
578 OsWRKY30 function as positive regulators of SA signaling pathway in rice (Koo *et*
579 *al.*, 2009; Han *et al.*, 2013). Given the strong effects of OsLRR-RLK1 on MPKs and
580 WRKYs found here, the regulation of JA, SA, and ET levels by OsLRR-RLK1 may
581 be achieved mainly through MPK cascades and WRKYs.

582 In Arabidopsis, *pepr1pepr2* double mutants display reduced resistance to *S.*
583 *litorralis* (Klauser *et al.*, 2015), and *bak1* mutant plants are compromised in immunity
584 to aphids (Prince *et al.*, 2014). Here our experiments show that silencing of
585 *OsLRR-RLK1* decreased the TrypPIs activity and the resistance of rice to SSB larvae,
586 possibly via the impaired JA signaling (Fig. 7). This finding is consistent with our
587 previous results showing that as-*lox* plants, which had lower elicited JA levels, were
588 susceptible to SSB attack (Zhou *et al.*, 2009). Previous studies have also demonstrated
589 that the ET signaling pathway positively regulates rice resistance to SSB: antisense
590 expression of *OsACS2* (as-*acs2*) reduced herbivore-induced ET emission and the
591 resistance of rice to SSB (Lu *et al.*, 2014). Therefore, we propose that the
592 compromised resistance of ir-*lrr* lines to SSB is a result of low JA and ET levels,

593 which are positively mediated by OsLRR-RLK1.

594 In summary, our results demonstrate that OsLRR-RLK1 functions as a potential
595 herbivore-recognition receptor of rice, and initiates induced defenses against SSB. We
596 propose that the membrane-localized OsLRR-RLK1 may either directly bind to
597 HAMPs or indirectly bind to other HAMP-induced early signaling molecules and
598 immediately activate MPKs, which subsequently increase the activity of downstream
599 WRKYs. Then, the activated MPKs and WRKYs regulate the biosynthesis of
600 herbivore-related phytohormones, including JA, SA and ET, which result in effective
601 induced defense responses against SSB. Our findings show how a plant employs an
602 early responsive LRR-RLK to trigger specific defense responses against herbivores.
603 We propose OsLRR-RLK1 as a candidate receptor of early signaling molecules that
604 are associated with herbivory.

605

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618

619 **Author Contributions**

620 L. H., M. Y., M. E. and Y. L. designed the research; L. H., M. Y., P. K., and M. Y.
621 performed experiments; L. H., M. Y., P. K., M. Y., M. E. and Y. L. analyzed and
622 interpreted data; L. H., M. Y., M. E., and Y. L. prepared and wrote the article. All

623 authors read and approved the manuscript.

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Figure Legends

Fig. 1. Subcellular localization of OsLRR-RLK1.

Agrobacterium-mediated transient expression in *Nicotiana benthamiana* leaves of AtPIP2A-mCherry and OsLRR-RLK1-enhanced yellow fluorescent protein (EYFP). The first column shows mCherry fluorescence, and the second column shows the EYFP fluorescence. Overlaid image indicates co-localization of AtPIP2A-mCherry and OsLRR-RLK1-EYFP. White square in the overlaid image is shown as a detailed picture which is magnified in the fourth column. Yellow line in the detailed picture indicates the region of interest (ROI) that corresponds to the intensity profile in the last column. Intensity profile indicates the gray value of pixels across the ROI in the mCherry and EYFP channels. Leaf epidermal cells were imaged by confocal microscopy 72 h after infiltration with a suspension of each *Agrobacterium tumefaciens* strain at an OD_{600} = 0.7. Scale bars: 20 μm.

Fig. 2. Transcriptional regulation of *OsLRR-RLK1*.

Mean transcript levels (+SE, n = 5) of *OsLRR-RLK1* in rice stems that were infested by rice striped stem borer (SSB, a), mechanically wounded (W, b), treated by *Spodoptera frugiperda* oral secretions (OS) after wounding (W + *S. frugiperda* OS, c), or jasmonic acid (JA, d). Con, control plants; Buf, buffer. Transcript levels were analyzed by quantitative real-time PCR. Asterisks represent significant differences between treatments and controls at the indicated times (Two-way analysis of variance [ANOVA], followed by pairwise comparisons of Least Squares Means [LSMeans], P values were corrected by False Discovery Rate [FDR] method; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Fig. 3. *OsLRR-RLK1* acts upstream of MPK cascades.

(a) MPK activation in ir-*lrr* lines and wild-type (WT) plants, which were infested by a third-instar striped stem borer (SSB) larva. Infested stems from five replicate plants were harvested at indicated times. Immunoblotting was performed using either anti-pTEpY antibody (upper panel) to detect phosphorylated MPKs, or actin antibody (lower panel) as a loading control which was detected on a replicate blot. For

quantification of immunodetection signals, see Fig. S10. This experiment was repeated three times, and the effect of *OsLRR-RLK1* was consistently observed (Fig. S10). (b to d) Mean transcript levels (+SE, n = 5) of *OsMPK3* (b), *OsMEK4* (c) and *OsMPK6* (d) in ir-*lrr* lines and WT plants that were individually infested by a third-instar SSB larva. (e, f) Mean transcript levels (+SE, n = 5) of *OsLRR-RLK1* in as-*mpk3* (e), as-*mpk6* (f) and WT plants that were individually infested by a third-instar SSB larva. Asterisks represent significant differences between ir-*lrr* lines and WT plants at indicated times (Two-way analysis of variance [ANOVA], followed by pairwise comparisons of Least Squares Means [LSMeans], P values were corrected by False Discovery Rate [FDR] method; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Fig. 4. *OsLRR-RLK1* regulates defense-related WRKY transcription factors.

Mean transcript levels (+SE, n = 5) of *OsWRKY70* (a), *OsWRKY53* (b), *OsWRKY45* (c), *OsWRKY24* (d), *OsWRKY30* (e) and *OsWRKY33* (f) in ir-*lrr* lines and wild-type (WT) plants that were individually infested by a third-instar striped stem borer larva. Asterisks represent significant differences between ir-*lrr* lines and WT plants at indicated times (Two-way analysis of variance [ANOVA], followed by pairwise comparisons of Least Squares Means [LSMeans], P values were corrected by False Discovery Rate [FDR] method; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Fig. 5. OsLRR-RLK1 mediates herbivore-induced jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) biosynthesis.

(a, b) Mean levels (+SE, n = 5) of JA (a) and JA-Ile (b) in ir-*lrr* lines and wild-type (WT) plants that were individually infested by a third-instar striped stem borer (SSB) larva. (c, d) Mean transcript levels (+SE, n = 5) of *OsHI-LOX* (c) and *OsAOS1* (d) in ir-*lrr* lines and WT plants that were individually infested by a third-instar SSB larva. (e) Mean levels (+SE, n = 5) of ET in ir-*lrr* lines and WT plants that were individually infested by a third-instar SSB larva. (f) Mean transcript levels (+SE, n = 5) of *OsACS2* in ir-*lrr* lines and WT plants that were individually infested by a third-instar SSB larva. (g) Mean levels (+SE, n = 5) of SA in ir-*lrr* lines and WT plants that were individually infested by a third-instar SSB larva. (h) Mean transcript levels (+SE, n = 5) of SA biosynthesis-related gene *OsICS1* in ir-*lrr* lines and WT plants that were

individually infested by a third-instar SSB larva. FW, fresh weight. Asterisks represent significant differences between ir-*lrr* lines and WT plants at indicated times (Two-way analysis of variance [ANOVA], followed by pairwise comparisons of Least Squares Means [LSMeans], *P* values were corrected by False Discovery Rate [FDR] method; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Fig. 6. *OsLRR-RLK1* is not regulated by defense hormone signaling cascades.

Mean transcript levels (+SE, $n = 5$) of *OsLRR-RLK1* in as-*lox* (a), as-*aos1* (b), as-*ics1* (c), as-*acs2* (d) lines and wild-type (WT) plants that were individually infested by a third-instar striped stem borer larva.

Fig. 7. Silencing of *OsLRR-RLK1* attenuates trypsin protease inhibitor (TrypPI) activity and rice resistance to the striped stem borer (SSB).

(a) Mean TrypPI activity (+SE, $n = 5$) in ir-*lrr* lines and wild-type (WT) plants that were individually infested by a third-instar SSB larva for 3 days. (b) Mean larval weight (+SE, $n = 30$) of SSB feeding on ir-*lrr* lines or WT plants for 12 days. Letters indicate significant differences between lines (one way-analysis of variance [ANOVA], followed by multiple comparisons of Least Squares Means [LSMeans], which were corrected using False Discovery Rate [FDR] method, $P < 0.05$). (c) Mean activity (+SE, $n = 5$) of TrypPIs in ir-1 line and WT plants which were individually treated with 100 μg of methyl jasmonate (MeJA) in 20 μl of lanolin paste (+MeJA) followed by a SSB larva feeding for 3 days. (d) Mean larval weight (+SE, $n = 30$) of SSB larvae 12 d after feeding on ir-1 and WT plants that were individually treated with 100 μg of MeJA in 20 μl of lanolin paste (+MeJA). (e) Mean activity (+SE, $n = 5$) of TrypPIs in ir-1 line and WT plants which were individually treated with 20 μl of pure lanolin paste (+Lanolin) followed by a SSB larva feeding for 3 days. (f) Mean larval weight (+SE , $n = 30$) of SSB larvae 12 d after feeding on ir-1 and WT plants that were individually treated with 20 μl of pure lanolin paste (+Lanolin). Asterisks represent significant differences between ir-1 and WT plants (Student's *t* tests, **, $P < 0.01$). (g) Damaged phenotypes of ir-*lrr* lines and WT plants that were individually infested by a third-instar SSB larva for 7 days ($n = 20$).

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Experimental setup used to infest rice plants with striped stem borer (SSB) larvae.

Fig. S2 Transformation vector used in this study.

Fig. S3 Nucleotide sequence and the deduced amino acid sequence of *OsLRR-RLK1*.

Fig. S4 Phylogenetic analysis of defense-related leucine rich repeat receptor-like kinases from Arabidopsis, tobacco and rice.

Fig. S5 Protein alignment of OsLRR-RLK1 with homologous proteins in Arabidopsis.

Fig. S6 Salicylic acid (SA) treatment does not induce the expression of *OsLRR-RLK1*.

Fig. S7 DNA gel-blot analysis of ir-*lrr* and wild-type (WT) plants.

Fig. S8 Reduction of *OsLRR-RLK1* does not co-silence the transcript levels of its highly similar genes.

Fig. S9 Growth phenotypes of ir-*lrr* and wild-type (WT) plants.

Fig. S10 Relative activation of OsMPK3 and OsMPK6 in ir-*lrr* and wild-type (WT) plants.

Fig. S11 OsLRR-RLK1 does not regulate wound-elicited OsMPK3 and OsMPK6 activation and the levels of JA and SA.

Table S1 Primers and probes used for QRT-PCR of target genes.